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Award Number: W81XWH-06-1-0393

TITLE: Investigation of Rho signaling pathways in 3D collagen matrices via multidimensional microscopy and visualization techniques

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REPORT DATE: March 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01/03/07		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 1 Mar 2006 – 28 Feb 2007	
4. TITLE AND SUBTITLE  Investigation of Rho signaling pathways in 3D collagen matrices via multidimensional microscopy and visualization techniques				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0393	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Steven Trier  E-Mail: <a href="mailto:smtrier@wisc.edu">smtrier@wisc.edu</a>				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  University of Wisconsin Madison, WI 53715				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: Spatial-temporal dynamics of proteins involved in cell-cell communication, cell-extracellular matrix interactions, and ultimately tissue organization are difficult to study using conventional biochemical approaches. Recent progress in the development of 3D culture models has provided a more physiologically relevant growth environment, in which breast cancer cells imbedded within floating collagen matrices undergo morphogenesis, in part, through contraction of the surrounding matrix. The importance of rho kinase (ROCK) generated contractility in this process has previously been demonstrated through antibody staining of cells imbedded in collagen matrices of differing rigidities and treatment with pharmacological ROCK inhibitors. We are stably transfecting T47D human breast cancer cells, cultured within floating collagen matrices, with fluorescent fusion proteins, and observing them through the course of morphogenesis (5-11 days). Spectral Lifetime Imaging Microscopy (SLIM) is used to separate second harmonic generated (SHG) signals from intrinsic and extrinsic fluorescence signals. Together these multidimensional signals map targeted regions of intercellular protein environment and their interaction with the extracellular matrix and can be applied to further studies of cell adhesion and motility.					
15. SUBJECT TERMS No subject terms provided					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	6	19b. TELEPHONE NUMBER (include area code)

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## INTRODUCTION:

Increased breast density is correlated to a 4-6 fold increased risk in developing breast cancer, yet the physical and molecular mechanisms are poorly understood. Three-dimensional (3D) culture and tissue models have previously shown to recapitulate these phenomena in vitro, in which an important molecular regulator is believed to be through Rho related signaling pathways. This grant is aimed at using fluorescence lifetime imaging (FLIM) combined with multi-photon laser scanning microscopy (MPLSM) to increase the understanding into how contractility and breast cancer progression are related. In particular, Rho has previously been shown to play a role in cell contractility within 3D collagen cultures [1]. Fluorescence signatures of breast epithelial cells transfected with GFP tagged RhoA and its effector molecules are being studied for differences in intensity and lifetime, as spatial-temporal indicators of the local protein environment and activation state. Furthering these measurements, the analogous pairs of Rho and effector molecules have been transected with a second fluorophore to study FRET interactions via FLIM methods. Combined Spectral-lifetime imaging (SLIM) will be used to distinguish between the fluorescence emitted from intrinsic sources, such as collagen, NADH and FAD, as well as multiple fluorophores in FRET populations.

## BODY:

In the first year of this grant I have learned a great deal to ensure the project's continued success and my progress towards my thesis research. The multidisciplinary training and information needed for this project has been a rewarding experience that I am lucky to be a part of as a graduate student at the Laboratory for Optical and Computational Instrumentation (LOCI). LOCI is headed by Kevin Eliceiri, Patti Keely and John White and they have helped me become trained in the various disciplines needed for this project's continued success, using biological, microscopy and engineering approaches. This has taken some training aside from the traditional biomedical engineering approaches but I feel I am now at the position to critically think through a biomedical problem across disciplines.

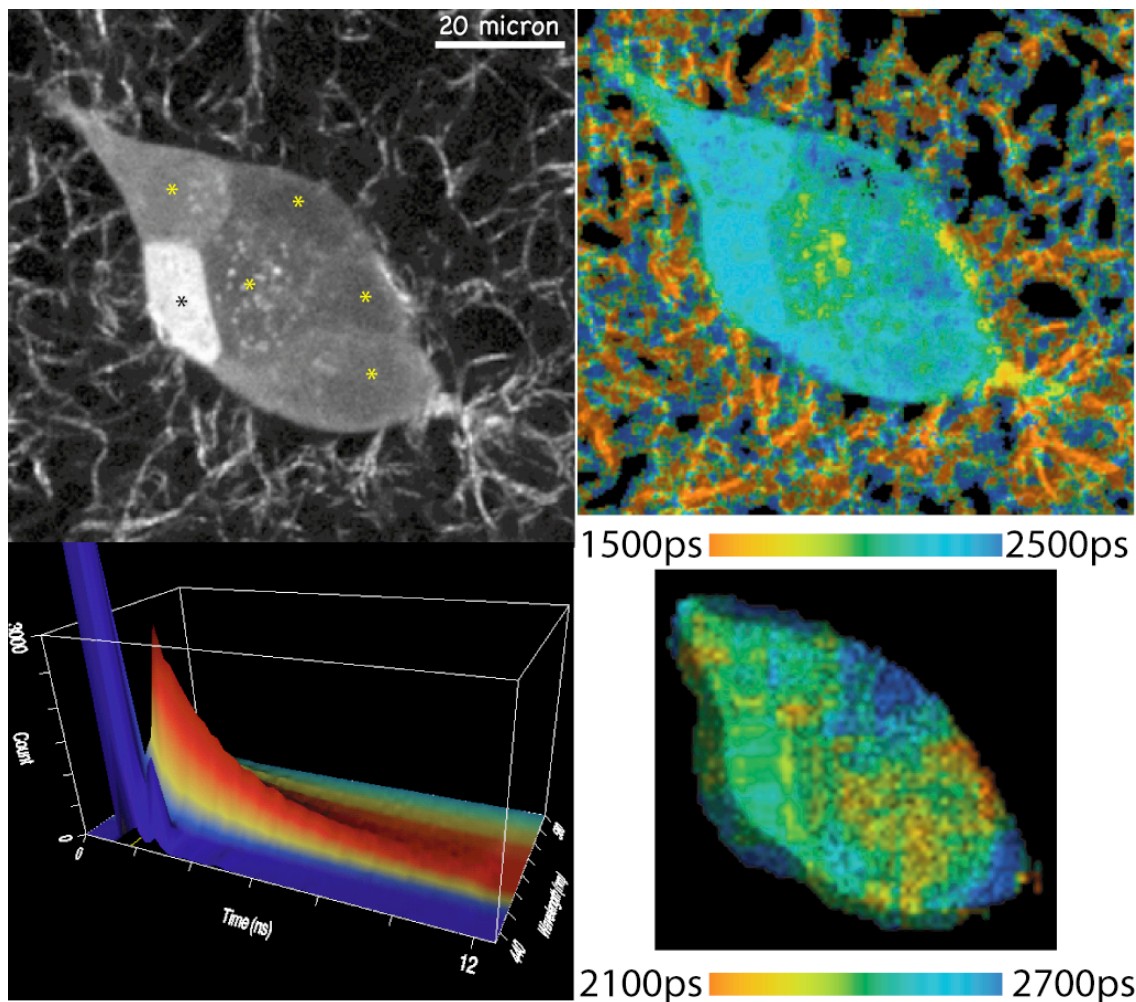
Suzanne Ponik, a postdoctoral fellow in the Keely Lab at the UW-Madison, has trained me in 3D cell culture and helped create stable T47D and NMuMG breast cell lines expressing GFP variants. Cell lines expressing Rho-GFP, Rhotekin (RBD)-GFP, dominant active (63L) Rho- GFP and GFP alone have been selected and sorted twice by flow cytometry. Aliquots of these cells have been frozen down and are available for experimentation.

Importantly for the aims of my proposal, the expression of GFP-Rho constructs is maintained in 3D culture, so we are now ready to use this system as a tool. As the first step, I have begun to characterize by fluorescent lifetime imaging (FLIM) the localization and activation of GFP-Rho probes across models for density and rigidity. To thoroughly understand cellular response to three-dimensional cues, a temporal understanding of specific regions will be necessary. To aid in these measurements micro-scale cultures are being constructed, such that regions of a well defined culture model may be imaged through proliferation and differentiation.

FLIM and SLIM mapping of day four T47D cells expressing GFP-RBD show areas of shortened GFP lifetime at membrane areas of collagen interaction and also an internal vesicle pool. In addition, double-transfected, mOrange FRET pairs have been made in the T47D lines and their activities are actively being characterized using these same approaches. Combinations of all

constructs and controls are being established to thoroughly define this model system. Resulting from this analysis should be a thorough understanding of FRET interactions and FLIM analysis, such that it can become a turnkey technology for the LOCI community. If mOrange proves to be problematic, a second fluorophore, mCherry, has also been obtained from Roger Tsien's laboratory at the University of California at San Diego.

My colleague and fellow DOD fellow Long Yan has helped to train me on the spectral-lifetime imaging system and in the analysis of lifetime images. One of the more difficult objectives in this study has been in interpreting and representing the data in a quantitative manner. Through logical analysis sequences it has possible to identify and isolate heterogeneity in lifetime signatures across the different populations. Intensity profiles through time and space as well as spectral and lifetime signatures are providing the tools expected for precise protein localization and activity studies (Figure 1). A deeper understanding and more rigorous testing is necessary before any conclusions can be accurately drawn between the biological, imaging, and computational aspects of this work.



**Figure 1: Day 4 T47D cells stably expressing and RBD-GFP fusion protein intensity (upper left), lifetime (upper right). The peak of the GFP signal, as observed in red in the lower left was filtered and analyzed independently for changes in lifetime (lower right).**

LOCI senior programmer, Curtis Rueden's expertise in image analysis has been instrumental in the most difficult aspect of this project; quantification and representation of processed information in an efficient manner. This is adding significantly to the quantitative engineering training I have already received. His computational analysis program, SlimPlotter has been developed with my input to help analyze spectral-lifetime datasets, as well as the beginnings of intensity analysis in ImageJ and VisBio

During this year I have also collaborated with Professor David Beebe's lab in Biomedical Engineering here at the UW-Madison. My initial training had taught me to think about defining local 3D microenvironments, and the microfabrication tools that are available. This collaboration is providing a link between bioengineers, biology and biophotonics clusters with aims at precisely manipulating cells and tissues, while observing and modulating subcellular phenomena. This combination is crucial for me to be adequately trained at the engineering/breast cancer interface.

#### KEY RESEARCH ACCOMPLISHMENTS:

- Proficient in 3D cell culture
- Establishment of GFP-Rho, GFP-RBD, GFP-DARho Cell lines (T47D, NMuMG)
- Establishment of FRET cell lines, and controls, with mOrange (T47D)
- Proficient in Spectral-Lifetime imaging
- Proficient in lifetime data analysis
- Characterization of lifetime signatures among the different constructs
- Beginning Intensity based analysis

#### REPORTABLE OUTCOMES:

Steve Trier, Suzanne Ponik, Long Yan, Paolo Provenzano, Kraig Kumfer, Kevin Eliceiri, John White, Patricia Keely. "Investigation of Signaling in 3D Breast Cancer Models with Spectral Lifetime Multiphoton Microscopy." The International Society for Optical Engineering, BIOS 2007, San Jose, California, January 2007.

#### CONCLUSION:

This first year has been important in the progress and maturation of my approach and my science. I have become a member of the LOCI community as well as the Keely lab. The lab members surrounding me have been great in helping me to the point I am at, and without their prior knowledge none of this would have come together so quickly. The implications of this research are vast. They may help to enable better breast cancer diagnosis and treatment using light based approaches, as well as provide new tools for studying molecular signatures in relevant tissue models. Advancement of in vitro and in vivo models, ex vivo imaging, and analysis should follow in the next grant year to fulfill and hopefully exceed the goals initially outlined.

#### REFERENCES:

1. M.A. Wozniak et al., Journal of Cell Biology 163 (3):583. 2003.